Neuromedin U Depolarizes Rat Hypothalamic Paraventricular Nucleus Neurons In Vitro by Enhancing $I_H$ Channel Activity

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Submitted 10 March 2003; accepted in final form 11 April 2003

Qiu, De-Lai, Chun-Ping Chu, Tetsuro Shirasaka, Takashi Nabekura, Takato Kunitake, Kazuo Kato, Masamitsu Nakazato, Takahiko Katoh, and Hiroshi Kannan. Neuromedin U depolarizes rat hypothalamic paraventricular nucleus neurons in vitro by enhancing $I_H$ channel activity. J Neurophysiol 90: 843–850, 2003; 10.1152/jn.00225.2003. The effect of neuromedin U (NMU) on rat paraventricular nucleus (PVN) neurons was examined using whole cell patch-clamp recordings. Under current-clamp, 31% of PVN parvocellular neurons ($n = 243$) were depolarized by 100 nM NMU, but magnocellular neurons were not affected. NMU (10 nM to 1 μM) resulted in increased basal firing rate and depolarization in a dose-dependent manner with an EC50 of 70 nM. NMU-induced depolarization was unaffected by co-perfusion with 0.5/1001 H9262 arachidonic acid metabolite release, and intracellular Ca2+ (Raddatz et al. 2000). NMU induces extracellular acidification, the paraventricular nucleus (PVN) of the rat hypothalamus and has potent effects on smooth muscle. The two receptors for the gut and CNS (Ballesta et al. 1988; Minamino et al. 1985) are G protein-coupled receptors (GPCRs) (Howard et al. 2000). NMU-R2 is expressed in the paraventricular nucleus (PVN) of the rat hypothalamus (Raddatz et al. 2000). NMU induces extracellular acidification, arachidonic acid metabolite release, and intracellular Ca2+ mobilization in cells expressing NMU-R1 or NMU-R2 (Howard et al. 2000; Shan et al. 2000). Recently, we found that intracerebroventricular (icv) application of 70 μM ZD 7288 completely inhibited NMU-induced depolarization. Under voltage-clamp, 1 μM NMU produced negligible inward current but did increase the hyperpolarization-activated current ($I_h$) at step potentials less than −80 mV. The effects of NMU on $I_h$ were voltage-dependent, and NMU shifted the $I_h$ conductance-voltage relationship ($V_{1/2}$) by about 10.8 mV and enhanced $I_h$ kinetics without changing the slope constant ($k$). Extracellular application of 70 μM ZD 7288 or 3 mM Cs+ blocked $I_h$ and the effects of NMU in voltage-clamp. These results suggest that NMU selectively depolarizes the subpopulation of PVN parvocellular neurons via enhancement of the hyperpolarization-activated inward current.

METHODS

Hypothalamic slice preparation

Hypothalamic slices were prepared from P12- to P14-day-old male Wistar rats, as previously described (Shirasaka et al. 2001). All experiments were approved by the Ethics Committee of the Miyazaki Medical College and were in accordance with international guidelines on the ethical use of animals in laboratory experiments. Briefly, the brain was quickly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 140 NaCl, 3 KCl, 1.3 MgSO4, 1.4 NaH2PO4, 11 d-glucose, 5 HEPES, 2.4 CaCl2, and 3.25 NaOH. The pH was 7.3; the osmolality was 290–300 mOsm, and the fluid was bubbled with 100% O2. Coronal slices were 250 μm in thickness, including PVN, and were prepared using a vibrating brain slicer (DSK-2000; Doshaka, Kyoto, Japan). The slices were incubated for 1-11 h in a chamber filled with equilibrated ACSF at room temperature (24–26°C) before recordings were started.

Electrophysiology

Patch pipettes were made with a puller (PB-7; Narishige, Tokyo) from thick-wall borosilicate glass (GD-1.5; Narishige). They were filled with a solution consisting of (in mM) 130 potassium gluconate, 2.5 KCl, 1.2 MgCl2, 2.0 CaCl2, and 10 HEPES. The pH was 7.3; the osmolality was 280–330 mOsm, and the fluid was bubbled with 95% O2/5% CO2. The pipettes were filled with a solution consisting of (in mM) 130 potassium gluconate,
10 HEPES, 10 KCl, 1 CaCl₂, 5 EGTA, 1 MgCl₂, 2 Na₂ATP, and 0.5 Na₃GTP. The pH was adjusted to 7.2 with KOH. Patch pipette resistances were 5–7 MΩ in the bath, with series resistances in the range of 10–20 MΩ, compensated by 80%. The liquid junction potential (10 mV) was corrected for according to the method described by Neher (1992). Membrane potentials and/or currents were monitored with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), acquired through a Digidata 1200 series A/D interface on a personal computer using Clampex 7.0 software (Axon Instruments). Selected traces were saved to the hard drive of a computer, and all data were saved to a 4.7-GB DVD-RAM.

The membrane potential and current were low-pass-filtered at 1–5 kHz. Whole cell recordings were made from microscopically identified cells. Once stable recording conditions were obtained, a PVN neuron was identified electrophysiologically as type I (magnocellular) or type II (parvocellular) according to previously established criteria by current-clamp in standard ACSF (Luther et al. 2000). Type I neurons displayed transient outward rectification, while Type II did not. In voltage-clamp, TTX was routinely included in external recording solutions to block voltage-gated Na⁺ channels.

**Chemicals**

Reagents included rat NMU-23 (Peptide Institute, Japan), ZD 7288 (Tocris Cookson, Ballwin, MO), CsCl (Sigma, St. Louis, MO), and TTX (Sigma). ZD 7288 was prepared as a 50 mM stock solution (in H₂O) and stored at −20°C until use. The other drugs were dissolved in ACSF.

**Data analysis**

Data were analyzed using Clampfit 8.0 (Axon Instruments) and are expressed as mean ± SE. \( I_{\text{H}} \) was determined by subtracting \( I_{\text{Ins}} \) from \( I_{\text{ss}} \) at each hyperpolarizing voltage step using the equation

\[
I_{\text{H}} = I_{\text{ss}} - I_{\text{Ins}}
\]

and \( I_{\text{H}} \) conductance (\( G_{\text{H}} \)) was estimated as the amplitude of \( I_{\text{H}} \) measured at various potentials (\( V \)) divided by the driving force (\( V - E_{\text{H}} \)), where \( E_{\text{H}} \) is the reversal potential of \( I_{\text{H}} \) (Ghamari-Langroudi and Bourque 2000) as follows

\[
G_{\text{H}} = I_{\text{H}}/(V - E_{\text{H}})
\]

Differences between mean values recorded under control and test conditions were evaluated using Student’s t-test or one-way ANOVA with Tukey’s post-hoc test. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

**Neuronal membrane properties**

A total of 309 PVN neurons (66 type I, 243 type II) were sampled under whole cell current-clamp conditions. While none of the type I neurons showed a response to NMU, 76 of the type II neurons (31%) were sensitive to NMU. These neurons expressed a depolarizing response approximately 1 min after application of 100 nM NMU; specifically, they displayed time-dependent inward rectification during the hyperpolarizing pulses (Fig. 1, C and D) that was blocked by 70 μM ZD 7288 (Fig. 1B) or 3 mM Cs⁺ (not shown). These characteristics are consistent with \( I_{\text{H}} \) conductance (Ludwig et al. 1998; Santoro et al. 1998). Further, the responsive neurons exhibited a lack of transient outward rectification in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential (Fig. 1A) (Luther et al. 2000).

**FIG. 1.** Electrophysiological properties of paraventricular nucleus (PVN) neuromedin U (NMU)-sensitive neurons. A: the neuron displayed time-dependent inward rectification and lacked transient outward rectification (black arrow) in response to a series of depolarizing current pulses delivered at a hyperpolarized membrane potential. B: the neuron displayed inward rectification which was blocked by 70 μM ZD 7288. Holding potential was −60 mV. C: the neuron responded to a series of hyperpolarizing current pulses, expressing inward rectification. D: current-voltage relationships were obtained at the peak voltage (○) and at the steady-state voltage (●) during the hyperpolarizing pulses.
Effects of NMU on membrane potential

Applications of NMU in concentrations ranging from 10 nM to 1 μM NMU resulted in depolarization and increased firing rate in a concentration-dependent manner in NMU-sensitive PVN neurons when the holding potentials were −60 mV (Fig. 2); however, the characteristics of the action potentials were not changed (not shown). The depolarization response appeared approximately 40 s after NMU exposure and peaked at approximately 100 s, with a maximal depolarization range of 1.75 ± 0.47 to 7.18 ± 1.21 mV (Fig. 2C). The minimum NMU dose required to elicit an effect on membrane potential was 1 nM, and the maximum dose was approximately 1 μM. The EC50 was approximately 70 nM. This response was unaffected 1.21 mV in the presence of 1 M TTX, CNQX, and bicuculline following the application of 1 M NMU; mean ± SE, P > 0.05, n = 5).

Effects of NMU on membrane current

Application of 1 μM NMU to NMU-sensitive neurons with voltage-clamp at −60 mV produced a negligible inward current (7.5 ± 2.1 pA, n = 16). However, when neurons were held at −60 mV and a series of 1-s hyperpolarizing voltage steps were held from −60 to −150 mV, NMU induced a significant increment in steady-state current (Ist at step potentials less than −80 mV (Fig. 3, A and B, P < 0.05, n = 7) and instantaneous current (Iins) at step potentials less than −100 mV (Fig. 3, A and C, P < 0.05, n = 7). NMU-induced increases in Iins was not affected by Ba2+ (Fig. 4A), but was blocked by ZD 7288 (Fig. 5C) or Cs+ (not shown). Simultaneously, the net reversal potential of the NMU-sensitive component of the fast current (determined in the presence of 100 μM Ba2+) was compared with the reversal potential for Iins (also determined in the presence of 100 μM Ba2+) (Cardenas et al. 1999). As illustrated in Fig. 4, A and B, the reversal potential was −34.0 ± 2.6 mV (n = 4) under control and NMU conditions. This was similar to the reversal potential of −33.5 ± 2.1 mV (n = 4) obtained with another group of NMU-sensitive neurons for the portion of Iins which was increased by changing the holding potential from −60 to −80 mV in the presence of Ba2+ (Fig. 4, C and D). Thus the increase in Iins produced by the shift in holding potential likely reflects an increase in tonically open Iht channels (Mayer and Westbrook 1983).

Blockade of the effects of NMU by ZD 7288 or Cs+

In current clamp, NMU-induced depolarization was not affected by TTX (Fig. 5A). However, application of ZD 7288 induced slight hyperpolarization in NMU-sensitive PVN neu-
rons and abolished NMU-induced depolarization (Fig. 5B). NMU increased $I_{\text{ss}}$ and $I_{\text{ins}}$ according to hyperpolarizing pulses. To determine whether NMU-induced increase in $I_{\text{ss}}$ and $I_{\text{ins}}$ represented enhanced $I_H$ channels, ZD 7288 and Cs⁺ (not shown) were used as $I_H$ channel blockers (Harris and Constanti 1995; Maccaferri and McBain 1996; McCormick and Pape 1990; Pape 1996). ZD 7288 significantly blocked $I_{\text{ins}}$ and NMU-induced increments of $I_{\text{ins}}$ at step potentials less than $-80 \text{mV}$. The $I-V$ relationships were linear in the presence of ZD 7288 (Fig. 5, C and D). Further, ZD 7288 blocked $I_{\text{ss}}$ and NMU-induced increments of $I_{\text{ss}}$ at step potentials less than $-80 \text{mV}$. The $I-V$ relationships were also linear in the presence of ZD 7288 (Fig. 5, C and E). This finding indicates that NMU-sensitive neurons have $I_H$ channels, which produce hyperpolarization-activated ZD 7288-sensitive inward currents ($I_{\text{ins}}$) and are activated at RMP, thus producing ZD 7288 sensitive $I_{\text{ins}}$.

Effects of NMU on $I_H$

In this study, 1 μM NMU significantly enhanced $I_H$ activity at step potentials less than $-80 \text{mV}$, and the maximal effects were at step potentials of $-100$ to $-120 \text{mV}$ (Fig. 3D). Furthermore, we estimated the effect of NMU on $I_H$ conductance ($G_{\text{H}}$) (see METHODS). $G_{\text{H}}$ was obtained in the NMU-sensitive neurons as shown in Fig. 6A: following a step to $-120 \text{mV}$ (1-s duration), the membrane voltage was stepped in the range of $-110$ to $-50 \text{mV}$ (1-s duration, 10-mV increments) (Maccaferri and McBain 1996). The plot of the instantaneous current at each test potential yielded the fully activated $I-V$ relationship, which was linear (Fig. 6B). The extrapolated reversal potential ($E_{\text{H}}$) was $-33.1 \pm 1.8 \text{mV}$ (Fig. 6B, $n = 5$), similar to that previously reported for $E_{\text{H}}$ in other nervous preparations (Maccaferri and McBain 1996; McCormick and Pape 1990; Pape 1996). The mean $G_{\text{H-V}}$ relationships are shown in Fig. 6C. Note that NMU enhanced $I_H$ conductance at step potentials more negative than $-80 \text{mV}$ ($P < 0.05$, $n = 7$). Furthermore, the modified Boltzmann equation was used as follows

$$G_{\text{H(V)}} = \frac{1}{1 + e^{(V-V_{\text{H,0}})/k}}$$  \hspace{1cm} (3)

where $G_{\text{H(V)}}$ is the fraction of maximal $G_{\text{H}}$ observed at $V$, $k$ is the slope factor, and $V_{\text{H,0}}$ is the half-maximal voltage. The mean values were as follows: $V_{\text{H,0}} = -110.2 \pm 2.3 \text{mV}$, $k = 13.1 \pm 2.0$ in control, and $V_{\text{H,0}} = -99.5 \pm 3.4 \text{mV}$, $k = 11.5 \pm 1.9$ during the application of NMU. These data reveal that NMU produced a significant shift in $V_{\text{H,0}}$ to a more depolarized potential (Fig. 6C, insert; $P < 0.05$). The slope factor values were not altered by NMU ($P > 0.05$).

Effects of NMU on the kinetics of $I_H$ activation

The time course of activation of $I_H$ was obtained from an analysis of the rising phase of the NMU-induced $I_H$ current evoked by hyperpolarizing steps to various voltages. As shown in Fig. 6D, the $I_H$ current traces were fit to a single exponential function of the form $A_t = A_\infty(1 - e^{-t/\tau})$, where $A_t$ is the amplitude of $I_H$ at time $t$, $A_\infty$ is the amplitude of $I_H$ at a steady state, and $\tau$ is the activation time constant (Ghamari-Langroudi...
and Bourque 2000). Figure 6D₂ reveals the plots of the means ± SE (n = 7) against voltage steps. The τ of NMU-sensitive neurons decreased from 800 ms at −70 mV to 100 ms at −140 mV and exhibited fast kinetics. NMU enhanced \( I_H \) channels kinetics exhibiting decrements of τ at step potentials more negative than approximately −80 mV (\( P < 0.05 \) vs. ACSF, \( n = 7 \)).

**DISCUSSION**

This study demonstrated that NMU enhanced \( I_H \) channels activity leading to excitatory responses in a subpopulation of PVN type II neurons.

**Expression of \( I_H \) in PVN NMU-sensitive neurons**

In the present study, NMU-sensitive PVN neurons displayed a time-dependent strong inward rectification during hyperpolarizing pulses (Fig. 1, C and D) that was blocked by 70 \( \mu \)M ZD 7288 (Fig. 1B). Further, these neurons did not display transient outward rectification. These properties are consistent with \( I_H \) conductance produced by \( I_H \) channels (Ludwig et al. 1998; Luther et al. 2000; Santoro et al. 1998; Stern 2001; Tasker and Dudek 1991).

**NMU excited PVN NMU-sensitive neurons by enhanced \( I_H \)**

NMU evoked small depolarization and increased neuronal excitability. Several previous studies have demonstrated that enhancement of \( I_H \) results in increased neuronal excitability and responsiveness to excitatory input. This occurs via release of neurons from tonic hyperpolarizing synaptic input and via facilitation of action potential triggering by depolarizing input or counter-balancing after-hyperpolarizations following action potentials (Pape 1996; Pape and McCormick 1989; Yagi and Sumino 1998). The presence of \( I_H \) in magnocellular neurosecretory cells of rat supraoptic nucleus provides an excitatory drive that contributes to phasic and tonic firing (Ghamari-Langroudi and Bourque 2000). \( I_H \) plays a significant role in setting both the RMP and the baseline level of excitability of hippocampal GABAergic interneurons found in the stratum oriens of area CA1 (Lupica et al. 2001).

Several findings in the current study suggest that NMU excites PVN neurons via enhanced \( I_H \) channel activity. First, the RMP of NMU-sensitive neurons was approximately −58 mV (\( V_h = −60 \) mV), and the \( E_{\text{H}} \) was approximately −33 mV (Fig. 6B), suggesting that \( I_H \) channels are partially active at RMP (Yagi and Sumino 1998). NMU enhanced the activity of \( I_H \) channels at RMP, induced NMU-sensitive neurons depolarizing in current-clamp (Fig. 2), and evoked increments of \( I_{\text{Ins}} \) by hyperpolarizing steps (Fig. 3C). NMU-induced increment in \( I_{\text{Ins}} \) was almost completely blocked by ZD 7288 (Fig. 5C), but not by \( \text{Ba}^{2+} \) (Fig. 4, A and B). Thus this likely reflects an increase in tonically activated \( I_H \) (Mayer and Westbrook 1983). Second, in voltage clamp, NMU resulted in an increase of \( I_H \) current and enhanced channel kinetics at step potentials less than −80 mV. NMU also produced a significant shift in \( V_{1/2} \) to a more depolarized potential. Third, ZD 7288 completely blocked NMU-induced depolarization (Fig. 5B) and abolished the effects of NMU on the neurons in voltage clamp (Fig. 5, C,
Possibly mechanism of NMU action

NMU-R2 is expressed in the PVN of the rat hypothalamus (Howard et al. 2000), and HCN1 and HCN3 mRNA expression is highly enriched in the PVN (Monteggia et al. 2000). It is possible that PVN-NGM-sensitive PVN neurons contain $I_{\text{HH}}$ (HCNs) channels and NMU-R2. A key property of neuronal HCN channels is their regulation by neurotransmitters and hormones that act via cAMP, cGMP, or intracellular Ca$^{2+}$ (Pape 1996). The cAMP and cGMP affect HCN channels by directly interacting with the cyclic nucleotide-binding domain protein of the C-terminus (Ludwig et al. 1998). The increment of intracellular Ca$^{2+}$ in thalamic relay cells can result in modifications of $I_{\text{HH}}$ that are similar to those observed following increases in cAMP (Lüthi and McCormick 1998). NMU-R1 is coupled to phospholipase C stimulation via a $G_{q}$-type G protein, resulting in the release of the inositol phosphate (IP) second messenger and increased intracellular Ca$^{2+}$ in COS-7 cells (Raddatz et al. 2000). NMU-R2 is also coupled to the $G_{q}$ family of G proteins in cells, inducing a rapid increase in intracellular Ca$^{2+}$ (Shan et al. 2000). In thalamic neurons, transient increases in intracellular Ca$^{2+}$ appeared to cause a reversible augmentation of $I_{\text{HH}}$ attributable to the rapid, Ca$^{2+}$-dependent formation of cyclic nucleotides (Lüthi and McCormick 1998). The increment of intracellular Ca$^{2+}$ activates soluble guanylate cyclase, leading to increased cGMP levels (Kuzmiski and Macvicar 2001).

Thus we propose that NMU binds to NMU-R2, resulting in
increased intracellular calcium and cGMP. This leads to an increase in \( I_{\text{H}} \) current and neuronal excitation. This response may contribute to activation of autonomic centers in the brain stem and spinal cord that regulate MABP, HR, and plasma norepinephrine.

**D I S C L O S U R E S**

This work was supported in part by a Grant-in-Aid for Science Research (14370024) from the Ministry of Education, Science, Sports, and Culture, Japan, and the Japanese Center of Excellence Program (Section of Life Science). This study was also carried out as part of the “Ground Research Announcement for Space Utilization” promoted by the Japan Space Forum.

**R E F E R E N C E S**


**FIG. 6.** Effects of NMU on \( I_{\text{H}} \): A: the reversal potential of \( I_{\text{H}} \) was determined by clamping the NMU-sensitive neuron to −120 mV for 1 s and depolarizing in 10 mV at 1-s increments to −50 mV. B: mean (n = 6) instantaneous currents of \( I_{\text{H}} \) (● shown in A) were averaged with respect to membrane potential, and a linear regression was performed. The reversal potential of \( I_{\text{H}} \) \((E_{\text{H}})\) was about −33 mV. C: the instantaneous 
(\( I_{\text{H}} \)) data shown in Fig. 3D were converted into conductance \((G_{\text{H}})\) using the equation \( G_{\text{H}} = I_{\text{H}}/(V + 33) \) (\( V \) is the test voltage). The solid lines are the best fit through the data points using the Boltzmann equation \((\text{ACSF}, C) 1 \mu \text{M NMU} \), ○, n = 7). The mean values were as follows: \( V_{1/2} = -110.2 \pm 2.3 \text{ mV}, k = 13.1 \pm 1.9 \text{ in the control and } V_{1/2} = -99.3 \pm 3.4 \text{ mV}, k = 11.5 \pm 1.8 \) during the application of NMU. The insert is a bar graph summarizing the effect of 1 μM NMU on half-maximal voltage \((V_{1/2})\). Note that 1 μM NMU significantly reduced \( V_{1/2} \) and elicited a positive shift \( V_{1/2} \) by about 10.8 mV. All data are mean ± SE. *P < 0.05 vs. ACSF. D: \( I_{\text{H}} \) traces evoked by steps to various voltages in the ACSF and during the application of 1 μM NMU. Superimposed on each trace is an exponential fit of the data points (a solid line extending to the right). The time constant used in the fits (\( \tau \)) is indicated beside each trace. D2: plots of the mean ± SE (n = 7) \( I_{\text{H}} \) activation time constants against voltage steps. Note that NMU enhanced \( I_{\text{H}} \) kinetics at step potentials less than −80 mV. *P < 0.05 vs. ACSF.

*J Neurophysiol* • VOL 90 • AUGUST 2003 • www.jn.org


